The killer toxin of the halotolerant yeast *Candida nodaensis*

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The production and secretion of killer toxins is a widespread phenomenon in yeasts[1]. Several killer (K) systems have been investigated; some of them in species considered more or less halotolerant, and which killer phenotype display has been, in some cases, associated with the degree of salt-stress in the environment[2,3].

In order to clarify the possible relation between killer activity and salt-stress tolerance, 58 different yeast strains were assayed as to salt-stress resistance[4,5] and killer/sensitive phenotypes in the absence and in the presence of NaCl[5] . This survey allowed the identification of several strong salt/dependent killer phenotypes (Table I)[5].

Table I. Killer phenotype variation with salt concentration in the assay. Results are expressed as the percentage of killed strains, from the total assayed as sensitive, at each salt molarity. Only K strains which killed more than one strain were considered.

NaCl - Tolerance Class	K strains	[NaCl] in the assay (M)	S strains (%) 0	0.5	1	1.5	2	2.5	3	3.5
		Number of strains tested for S. phenotype (100%)	47	56	47	45	20	210	7	5
1 M	*P. jadinii*		38.6%	12.5%	0	n.a.	n.a.	n.a.	n.a.	n.a.
	Z. fermentans		5.3%	7.1%	6.4%	n.a.	n.a.	n.a.	n.a.	n.a.
2 M	*K. lactis* CBS 2359		26.3%	32.1%	4.3%	0	0	n.a.	n.a.	n.a.
	P. anomala IGC 4121		35.1%	48.2%	46.8%	44.4%	50.0%	n.a.	n.a.	n.a.
	S. cerevisiae IGC 4620		8.8%	8.9%	10.6%	4.4%	0	n.a.	n.a.	n.a.
3 M	*C. famata*		0	1.8%	21.3%	28.9%	30.0%	15.0%	0	n.a.
	D. hansenii		14.0%	23.2%	34.0%	35.6%	40.0%	35.0%	0	n.a.
	P. farinosa		10.5%	21.4%	21.3%	13.3%	5.0%	0	0	n.a.
	P. membranaefaciens IGC 3706		8.8%	3.6%	0	0	0	n.a.	n.a.	n.a.
4 M	***C. nodaensis***		**12.3%**	**25.2%**	**34.0%**	**35.6%**	**40.0%**	**25.0%**	0	0
	P. guilliermondii		5.3%	3.6%	4.3%	8.7%	10.0%	5.0%	0	0
	P. norbitaphila		5.3%	17.9%	17.0%	11.1%	0	0	0	0

Candida nodaensis, one of the strongest killer strains identified, has been selected for further killer toxin production and purification.

Isolation and preliminary characterization of *C. nodaensis* killer factor (Figs. 1, 2, 3, 4)

Growth at 25°C, 160 rpm
Early stationary phase

↓ Centrifugation
2', 7000 rpm, 4°C

↓ Filtration of culture supernatant
0.45 µm

↓ Precipitation of culture filtrate
Adition of 3/4 vol. ethanol

↓ Centrifugation
10', 7000 rpm, 0°C

↓ Liophilization

↓ Ressuspension in 10 mM CPB, pH 4.0
Ethanol precipitate

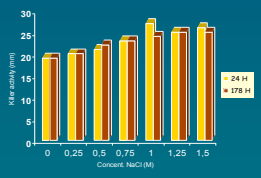

Fig. 1. Influence of NaCl (in the assay medium) on K activity.

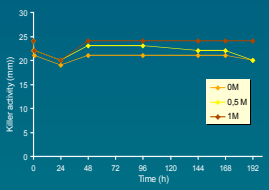

Fig. 2. Effect of NaCl on K toxin stability (the ethanol precipitate was resuspended in CPB with NaCl 0, 0.5 and 1M). K activity was assayed in the presence of 1M NaCl.

K activity was tested in methylene blue agar plates (YM-MB) either in the absence and in the presence of 0.5 and 1M NaCl.

Aliquots (50µl) of the sample to be tested were added to sterile disks previously placed on a top-agar layer of the sensitive strain *Pichia guilliermondii*.

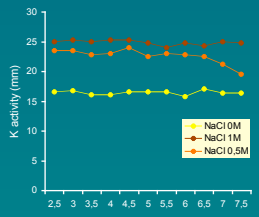

Fig. 3. Effect of pH on K toxin activity (K activity was assayed in the absence and in the presence of 0.5 and 1M NaCl).

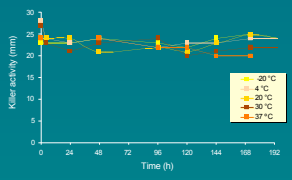

Fig. 4. K toxin activity after incubation of the ethanol precipitate at - 20°C, 4°C, 20°C, 30°C and 37°C. K activity was assayed in the presence of 1M NaCl.

Preliminary experiments performed to characterize *C. nodaensis* K factor renewed its interest for potential biotechnological applications. Thus, the following strategy was the purification of this yeast killer toxin.

Partial purification of the ethanol precipitate by Molecular Exclusion Chromatography

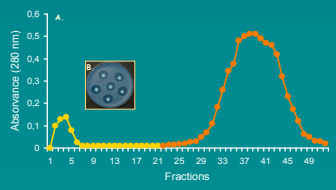

Fig. 5. A- Elution profile of K toxin through a Superdex 200 column (Pharmacia) equilibrated and eluted with CPB 200 Mm, pH 4.0 (fractions with K activity; fractions without K activity). B- Method used for detection of killer activity.

Active fractions were pooled, concentrated and its effect on sensitive cell viability was assessed in YEPD plates.

The percentage of S cells remaining alive after treatment with this K toxin solution was measured by comparison with CFU from a toxin-free control.

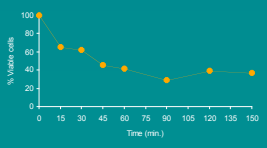

Fig. 6. Influence of partially purified K toxin on *P. guilliermondii* viability.

The majority of K toxins are considered labile proteins, unfitted for biotechnological purposes. Nevertheless, experiments performed to characterize *C. nodaensis* K factor showed that:

- It keeps its biological activity for long periods of time w/ or w/o high NaCl concentrations;
- It is stable after incubation in a relatively broad range of temperature (up to 37°C);
- It is stable after incubation in buffers covering a broad range of pH values (2.5 to 7.5).

Future strategies

Identification and isolation of K toxin receptor

Affinity Cromatography

Ionic Cromatography

Sequenciation

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(2) Llorente, P., Marquina, D. Santos, A., Peinado, J., M. and Spencer-Martins, I. (1997). **Appl. Environm. Microbiol. 63:** 1165-1167.

(3) Suzuki, C. (1999). **Yeast 15:** 123-131.

(4) Lages, F., Silva-Graça, M. and Lucas, C. (1999). **Microbiol. 145:** 2577-2586.

(5) Aguiar, C. and Lucas, C. (2000). **Food Technol. and Biotechnol. 38(1):** 39-46.